09622745
5000
SE 00/878

PCT/ SE 00 / 0 0 8 7 8

REC'D 27 JUN 2000
WIPO PCT

PRV

PATENT- OCH REGISTRERINGSVERKET
Patentavdelningen

4

Intyg
Certificate

Härmed intygas att bifogade kopior överensstämmer med de handlingar som ursprungligen ingivits till Patent- och registreringsverket i nedannämnda ansökan.

Ansökan ingavs ursprungligen på engelska.

This is to certify that the annexed is a true copy of the documents as originally filed with the Patent- and Registration Office in connection with the following patent application.

The application was originally filed in English.

(71) Sökande *Astra AB, Södertälje SE*
Applicant (s)

(21) Patentansökningsnummer *9901659-4*
Patent application number

(86) Ingivningsdatum *1999-05-06*
Date of filing

Stockholm, 2000-06-21

För Patent- och registreringsverket
For the Patent- and Registration Office

Görel Gustafsson

Avgift
Fee

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

PATENT- OCH REGISTRERINGSVERKET
SWEDEN

Postadress/Adress
Box 5055
S-102 42 STOCKHOLM

Telefon/Phone
+46 8 782 25 00
Vx 08-782 25 00

Telex
17978
PATOREG S

Telefax
+46 8 666 02 86
08-666 02 86

NEW METHODS

FIELD OF THE INVENTION

The present invention relates to nucleic acid molecules constituting $GABA_B$ receptor 1 promoters P1a and/or P1b, and to methods for screening for compounds which are modulators of $GABA_B$ receptor 1 transcription, said methods comprising the use of nucleic acid molecules constituting $GABA_B$ receptor P1a and/or P1b promoters.

BACKGROUND

$GABA_B$ receptor 1

GABA (γ-aminobutyric acid) is an endogenous neurotransmitter in the central and peripheral nervous systems. Receptors for GABA have traditionally been divided into $GABA_A$ and $GABA_B$ receptor subtypes. $GABA_B$ receptors (for a review see Kerr, D.I.B. and Ong, J. (1995) Pharmac. Ther. vol. 67, pp.187-246) belong to the superfamily of G-protein coupled receptors. $GABA_B$ receptor agonists are described as being of use in the treatment of CNS disorders, such as muscle relaxation in spinal spasticity, cardiovascular disorders, asthma, gut motility disorders such as irritable bowel syndrome and as prokinetic and anti-tussive agents. $GABA_B$ receptor agonists have also been disclosed as useful in the treatment of emesis (WO 96/11680) and reflux disease (WO 98/11885).

The cloning of the cDNA encoding the rat $GABA_B$ receptors splice isoforms $GABA_BR1a$ and $GABA_BR1b$ is disclosed by Kaupmann et al. (1997) Nature, vol. 386, 239-246. The mature rat $GABA_BR1b$ differed from $GABA_BR1a$ in that the N-terminal 147 residues were replaced by 18 different residues. It was presumed that the rat $GABA_BR1a$ and -b receptor variants are derived from the same gene by alternative splicing.

The cloning of the cDNA encoding the human $GABA_B$ receptor $GABA_BR1b$ is disclosed in WO 97/46675.

The cloning of the human $GABA_B$ receptor 1 gene and the elucidation of the exon-intron organization is in part or fully disclosed in PCT/SE98/01947, in EMBL HS271M21 (GenBank AL031983), EMBL AJ010170 to AJ010191, in Peters, HC et al., Neurogenetics 2; 47-54 (1998) and in Goei, VL et al. Biological Psychiatry. 44; 659-66 (1998). The human $GABA_B$ receptor 1 gene consists of 23 exons, spanning over a distance of 30 kb (Figure 1). The elucidation of the gene organization revealed that the human $GABA_BR1a$ and $GABA_BR1b$ are splice variants encoded by a single gene. The $GABA_BR1a$ and $GABA_BR1b$ isoforms are differentially expressed, at least in the rat (Kaupmann et al. (1997) Nature, vol. 386, 239-246). The physiological consequences of multiple $GABA_B$ receptor 1 splice isoforms has not yet been determined, but their existence constitute an opportunity for the development of specific pharmaceutical agents.

$GABA_B$ receptor 2

Based on its homology with the mammalian $GABA_BR1$ cDNA, a second member of the $GABA_B$ receptor family was identified (Jones, KA et al., Nature 396; 674-679 (1998), White, JA et al., Nature 396; 679-682 (1998), Kaupmann, K et al., Nature 396; 683-687 (1998), WO 99/20751). The corresponding protein, $GABA_BR2$, forms heteromers with $GABA_BR1a$ and R1b, resulting in cell surface expression of a functional GABA B receptor (Kuner, R et al. Science 283, 74-77 (1999)). At least in recombinant expression systems, $GABA_BR1$ and R2 coexpression is necessary for the formation of a functional $GABA_B$ receptor. Jones et al. (Nature 396; 674-679 (1998)) disclosed that a $GABA_BR1$: $GABA_BR2$ stochiometry of 1:1 is an optimal ratio for successful cell surface expression of a ligand binding and functional $GABA_B$ receptor. Thus, modulating $GABA_BR1$ expression could alter the stochiometry between $GABA_BR$ 1and other interacting proteins and be a means to regulate signaling through $GABA_B$ receptors and thereby interfere with various physiological processes.

Transcriptional regulation

Gene regulation is mediated by specific DNA elements in the promoter that directs binding of transcription factors, which thereby mediate transcription of the gene. Eukaryotic transcription factors can be divided in two main groups *i*) basal transcription factors that

interact with promoter sequences proximal to the start of transcription, thereby initiating transcription upon recruitment of RNA polymerase II and *ii*) transcription factors that bind to specific distal promoter elements, thereby modulating the transcription upon contact with the basal transcription machinery. The DNA sequence that directs the start of transcription in most eukaryotic genes is the TATA-box, which is often located approximately 30 base pairs upstream from the start of transcription. However, the TATA-box is not a prerequisite for initiation of transcription as there are many promoters, including the $GABA_B$ R1 promoters described in this study, that lack a TATA-box. A fundamental physiological process in the eukaryotic organism is that cells can communicate with their environment and respond to extracellular stimuli through signaling molecules, such as hormones and growth factors. The final event for such signaling is the binding of transcription factors to specific distal promoter elements leading to for example up-regulated or tissue specific gene expression. Because of their regulatory role, signaling molecules are putative targets for screening of therapeutic agents. The presence of two distinct and differentially regulated promoters within the human $GABA_B$ receptor 1 gene, disclosed in this patent application, makes it possible to screen for therapeutic agents selectively regulating expression of $GABA_B$ receptor 1a and 1b-type splice isoforms.

Indications

Compounds which are modulators of $GABA_B$ receptor 1 transcription are potentially useful in the treatment of disorders which are related to neurally-controlled physiological responses regulated by $GABA_B$ receptors, e.g. CNS disorders such as muscle relaxation in spinal spasticity, Alzheimer's disease and other dementias, psychiatric and neurological disorders such as depression, anxiety and epilepsy, cardiovascular disorders, asthma, gut motility disorders such as irritable bowel syndrome, emesis and reflux disease.

In some humans, the lower esophageal sphincter (LES) is prone to relaxing more frequently than in other humans. As a consequence, fluid from the stomach can pass into the esophagus since the mechanical barrier is temporarily lost at such times, an event hereinafter referred to as "reflux".

Gastro-esophageal reflux disease (GERD) is the most prevalent upper gastrointestinal tract disease. Current therapy has aimed at reducing gastric acid secretion, or by reducing esophageal acid exposure by enhancing esophageal clearance, lower esophageal sphincter tone and gastric emptying. The major mechanism behind reflux has been considered to depend on a hypotonic lower esophageal sphincter. However, recent research (e.g. Holloway & Dent (1990) Gastroenterol. Clin. N. Amer. 19, 517-535) has shown that most reflux episodes occur during transient lower esophageal sphincter relaxations (TLESR), i.e. relaxations not triggered by swallows. It has also been shown that gastric acid secretion usually is normal in patients with GERD. Consequently, there is a need for compounds which reduce the incidence of TLESR and thereby prevent reflux.

DESCRIPTION OF THE INVENTION

This invention relates to nucleic acid molecules constituting $GABA_B$ receptor 1 promoters and fragments of said promoters. By $GABA_B$ receptor 1 promoters is understood the nucleic acids sequences upstream of the ATG translation initiation codon of $GABA_B$ receptor 1a of the $GABA_B$ receptor 1 gene, designated P1a, and the nucleic acids sequences upstream of the ATG translation initiation codon of $GABA_B$ recpetor 1b of the $GABA_B$ receptor 1 gene, designated P1b, as illustrated in Figure 1.

In the present context the term "promoter" is meant to include core promoter sequences proximal to the start of transcription and upstream promoter elements which bind constitutively active transcription factors, as well as distal promoter elements which bind specific transcription factors.

Accordingly, the present invention provides a nucleic acid molecule constituting a human $GABA_B$ receptor 1 promoter P1a, or a functionally equivalent modified form thereof, or active fragments thereof. The present invention also provides a nucleic acid molecule constituting a human $GABA_B$ receptor 1 promoter P1b, or a functionally equivalent

modified form thereof, or active fragments thereof. By a functionally equivalent modified form is understood nucleic acids modified from the original sequence that can bind transcription factors. By active fragments of the promoters is understood nucleic acid fragments that can bind transcription factors.

In preferred forms of the invention the said nucleic acid molecule is selected from:
(a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 1;
(b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the DNA molecule as defined in (a).

In other preferred forms of the invention the said nucleic acid molecule is selected from:
(a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 2;
(b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the DNA molecule as defined in (a).

In another preferred form of the invention the said nucleic acid molecule may be a nucleic acid molecule constituting a human $GABA_B$ receptor 1 promoter P1a, or a functionally equivalent modified form thereof, or active fragments thereof, in combination with a nucleic acid molecule constituting a human $GABA_B$ receptor 1 promoter P1b, or a functionally equivalent modified form thereof, or active fragments thereof.

In another preferred form of the invention the said nucleic acid molecule may be a nucleic acid molecule selected from:
(a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 1;
(b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the DNA molecule as defined in (a);
in combination with a nucleic acid molecule is selected from:

(a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 2;
(b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the DNA molecule as defined in (a).

It should thus be understood that the nucleic acid molecules according to the invention is not to be limited strictly to molecules comprising the sequences set forth as SEQ ID : 1 and 2. Rather the invention encompasses nucleic acid molecules carrying modifications like substitutions, small deletions, insertions or inversions, which nevertheless have substantially the biochemical activity of the $GABA_B$ receptor promoters 1a and/or 1b according to the invention. Included in the invention are consequently nucleic acid molecules, the nucleotide sequence of which is at least 95% homologous, preferably at least 96%, 97%, 98% or 99% homologous, with the nucleotide sequence shown as SEQ ID NO: 1 or 2 in the Sequence Listing.

The term “stringent hybridization conditions” is known in the art from standard protocols (e.g. Current Protocols in Molecular Biology, editors F. Ausubel et al., John Wiley and Sons, Inc. 1994) and could be understood as as stringent or more stringent than those defined by e.g. hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at +65°C, and washing in 0.1xSSC / 0.1% SDS at +68°C.

It will be appreciated that the nucleic acid sequences shown in the Sequence Listing is only an example within a large but definite group of nucleic acid sequences which will have the $GABA_B$ receptor promoter activity.

In yet another aspect, the invention provides a vector transformed with a nucleic acid molecule of the present invention. The said vector can e.g. be a replicable expression vector which carries a nucleic acid molecule according to the invention. In the present context the term “replicable” means that the vector is able to replicate in a given type of host cell into which is has been introduced. Examples of vectors are viruses such as

bacteriophages, cosmids, plasmids and other recombination vectors. Nucleic acid molecules are inserted into vector genomes by methods well known in the art.

Another embodiment of the present invention is an expression system comprising nucleic acid molecules encoding $GABA_B$ receptor promoters P1a and/or P1b, or functionally equivalent modified forms, or active fragments thereof.

In preferred forms of this embodiment of the invention the said nucleic acid molecule is selected from: (a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 1 and/or SEQ ID NO: 2 ; (b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the polypeptide coding region of a DNA molecule as defined in (a).

In another preferred form of this embodiment of the invention the said nucleic acid molecule may be a nucleic acid molecule constituting a human $GABA_B$ receptor 1 promoter P1a, or a functionally equivalent modified form thereof, or active fragments thereof, in combination with a nucleic acid molecule constituting a human $GABA_B$ receptor 1 promoter P1b, or a functionally equivalent modified form thereof, or active fragments thereof.

In another preferred form of this embodiment of the invention the said nucleic acid molecule may be a nucleic acid molecule selected from:
(a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 1;
(b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the DNA molecule as defined in (a);
in combination with a nucleic acid molecule is selected from:
(a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 2;
(b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the DNA molecule as defined in (a).

The expression system may, in addition, comprise a reporter gene, the promoter and the reporter gene being positioned so that the expression of the reporter gene is regulated by the $GABA_B$ receptor 1 promoters P1a and/or P1b. Suitable expression systems according to the invention are e.g. bacterial or yeast plasmids, wide host range plasmids and vectors derived from combinations of plasmid and phage or virus DNA. Furthermore, an origin of replication and/or a dominant selection marker can be present in the vector according to the invention. Suitable reporter genes that can be used for the construction of expression systems according to the invention are e.g. the firefly luciferase gene, the bacterial chloramphenicol acetyl transferase (CAT) gene, the β-galactosidase (β-GAL) gene, and the green fluorescent protein (GFP).

A further aspect of this embodiment of the invention is a host cell transfected with an expression system comprising nucleic acid molecules constituting $GABA_B$ receptor promoters P1a and/or P1b, or functionally equivalent modified forms thereof, or active fragments thereof.

Suitable host cells are cells known to express $GABA_B$ receptors or cells known to express transcription factors which can influence the transcription of $GABA_B$ receptors. Host cells transfected with DNA encoding specific transcription factors can preferably be used to study the interaction with defined transcription factors and the $GABA_B$ receptor promoter.

Another embodiment of the invention is a method for the assay of $GABA_B$ receptor promoter activity said method comprising the use of a host cell transfected with an expression system comprising nucleic acid molecules constituting $GABA_B$ receptor promoters P1a and/or P1b, or functionally equivalent modified forms thereof, or active fragments thereof.

A further embodiment of the present invention is a method for the screening of compounds which are modulators of $GABA_B$ receptor 1 transcription, said method comprising the use of nucleic acid molecules constituting $GABA_B$ receptor P1a and/or P1b promoters.

Accordingly, the present invention provides a method for screening compounds which are modulators of $GABA_B$ receptor 1 transcription, comprising the steps of: (a) transfecting a cell host with a suitable expression system comprising a nucleic acid molecule constituting human $GABA_B$ receptor 1 promoter P1A, and/or a human $GABA_B$ receptor 1 promoter P1B or functionally equivalent modified forms, or active fragments thereof coupled to a reporter gene; (b) contacting a test compound with the cell; and (c) determining whether the test compound modulates the level of expression of the reporter gene.

A further embodiment of the invention is a transgenic non-human animal whose genome comprises an expression system comprising nucleic acid molecules constituting $GABA_B$ receptor promoters P1a and/or P1b, or functionally equivalent modified forms thereof, or active fragments thereof, coupled to a reporter gene.

Such transgenic non-human mammals can be generated by insertion of DNA comprising $GABA_B$ receptor promoters by microinjection, retroviral infection or other means well known to those skilled in the art, into appropriate fertilized embryos to produce a transgenic animal (Hogan B. et al Manipulating the Mouse Embryo, A Laboratory Manual, Cold Spring Harbor Laboratory (1986)).

Accordingly, the present invention provides a method for the screening of compounds which are modulators of $GABA_B$ receptor 1 transcription, comprising the use of a transgenic non-human animal whose genome comprises an expression system comprising nucleic acid molecules constituting $GABA_B$ receptor promoters P1a and/or P1b, or functionally equivalent modified forms thereof, or active fragments thereof, coupled to a reporter gene, or tissues or cells isolated from such transgenic animals.

EXAMPLES

The following examples are preferred non-limiting examples embodying preferred aspects of the invention.

Example 1. Isolation and identification of human $GABA_B$ R1 promoters P1a and P1b

Genomic DNA containing the human $GABA_B$ receptor gene was isolated from human genomic libraries and genomic DNA. Human genomic libraries were obtained from Clontech (Palo Alto, CA, USA). The libraries were constructed from female leukocyte DNA (catalogue # HL1111J), cloned into λEMBL-3 vector. The average size of inserts are 16 kb and the number of independent clones are 1.7×10^6. Human genomic DNA was obtained from Clontech (catalogue # 6550-1). In order to isolate recombinant phases containing exon and intron sequences of the human $GABA_B$ receptor gene, 48 individual bacterial plates with a diameter of 150 mm and approximately 4×10^4 individual plaques per plate, were screened. The methods and solutions used were as described in the Library Protocol Handbook: General Procedures for the Hybridization of Lambda Phage Libraries w/DNA Probes (Clontech) with some modifications as will be apparent from the following.

The experiment was carried out essentially as follows. The numbers are given per plate basis. A sample of the phage library diluted in 0.1 ml sterile lambda diluent was prepared in order to obtain an estimated titer of 40,000 pfu (plaque forming units). A 0.6 ml LB-medium culture of the *E. coli* host strain K802 (obtained from Clontech) was infected with 40000 pfu recombinant phages for 15 minutes at 37°C. The culture was then mixed with 7 ml top agarose (6.5 g of agarose added per liter LB) and poured onto LB plates. The plates were incubated at 37°C for approximately 7 hours. The plates were then chilled at +4°C.

Plaque hybridization experiments were as follows. Membrane filters, Colony/Plaque Screen (DuPont, Wilmington, DE, USA), were placed onto the top of the plates for 3

minutes. For denaturation of DNA the filters were removed and floated in 0.5 M NaOH on a plastic wrap for 2 minutes, with the plaque side up. This step was repeated once to ensure efficient denaturation. Following neutralization the membrane filters were placed in 1M Tris-HCl pH 7.5, two times 2 minutes and allowed to dry.

To obtain probes for DNA hybridization screening of the membrane filters, a $GABA_B$ receptor cDNA clone was digested with SacII and a 479 bp fragment, separated by agarose electrophoresis, excised and transferred to a polypropylene microcentrifuge tube. Additional probes were obtained by PCR amplification of various regions of the $GABA_B$ receptor cDNA. The isolated cDNA fragment was ^{32}P-labeled using Megaprime DNA labeling system (Amersham Pharmacia Biotech, Uppsala, Sweden) by the following procedure. Water was added at a ratio of 3 ml per gram of gel, and placed in a boiling water bath for 7 minutes to melt the gel and denature the DNA. A volume of DNA/agarose solution containing 25 ng of DNA was added to the labeling reaction, according to the supplier's instructions. Labeled nucleotides were removed from DNA labeling reactions using MicroSpin™ G-50 Columns (Amersham Pharmacia Biotech, Uppsala, Sweden).

The DNA hybridization reaction was performed under stringent conditions according to the method described below. The filter membranes were prehybridized at 65°C for at least 1 hour in a solution composed of 1% SDS, 1M NaCl, and 10% dextran sulfate using a hybridization oven (Hybaid Ltd, Ashford, UK). Following prehybridization a solution containing denatured herring sperm DNA of a final concentration of 100 µg/ml and the ^{32}P-labeled DNA probe at a concentration <10 ng/ml (for optimal signal to background ratio) was added to the prehybridization solution and the membrane filters were incubated at 65°C for 10-20 hours. Following the removal of the hybridization solution the membrane filters were first washed in a 2xSSC (0.3M NaCl, 0.03M Na-citrate), 1% SDS solution two times for 5 minutes at room temperature. In the next step, the membrane filters were incubated at 60°C two times for 30 minutes each in the same solution. In a third step, the filters were washed two times at room temperature in 0.1xSSC. Finally, the membrane filters were placed on a sheet of filter paper with the DNA face up, and allowed to dry. The dried membrane filters were then exposed to X-ray films and autoradiographed.

Of the approximately $2x10^6$ individual plaques analyzed, four hybridizing plaques were detected and isolated. These four isolates were designated #GR1, #GR12, #GR13 and #GR41, respectively. After several rescreening experiments, the recombinant phage DNA was purified using Qiagen Lambda Midi Kit (Qiagen GmbH, Germany). The purified DNA was digested with SalI and the fragments representing the inserts were isolated by agarose electrophoresis.

The 16kb insert of isolate #GR13 was cloned into SalI digested linearized pUC19, resulting in the plasmid pAM364. The insert was analyzed by PCR, restriction mapping and hybridization to ^{32}P-labeled DNA fragments representing various regions of the $GABA_B$ receptor cDNA.

The cloned fragment in the plasmid pAM364 was characterized by restriction enzyme mapping, using EcoRI, HindIII, PstI, and BamHI. The approximate positions of the exons and the approximate size of the introns were analyzed and determined by PCR-based exon-exon linking and agarose gel electrophoresis.

In order to facilitate nucleotide sequence analysis, 7 restriction sub-fragments derived from pAM364 were isolated and cloned individually into pUC19. The following strategy was employed; by combining PCR primers located within the pUC19 sequence either upstream or downstream of the cloning site, with a PCR primer with defined orientation and specific for the $GABA_B$ receptor derived subcloned fragment allowed the sequence determination.

The inserts were subjected to nucleotide sequence analysis. The nucleotide sequences for all subclones were determined using a Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham Pharmacia Biotech, Uppsala, Sweden). As primers for sequencing reactions specific oligonucleotides complementary to pUC19 or primers complementary to the human $GABA_B$ receptor cDNA were used.

The sequence of the human $GABA_B$ receptor gene fragment cloned in the plasmid pAM364 has previously been revealed (see PCT/SE98/01947). This genomic fragment was shown to contain the complete exons 1-11 and the complete introns 1-10 of the human $GABA_B$ receptor gene as well as > 3kb sequence upstream of exon 1. The elucidation of the gene organization revealed that the human $GABA_BR1a$ and $GABA_BR1b$ are splice variants encoded by a single gene.

In order to localize the putative human $GABA_B$ receptor promoter, we investigated the genomic sequence for the presence of consensus sequences of known regulatory promoter elements. To our surprise, we found that promoter elements, clustered in two regions: one region upstream of exon 1, and the other region in intron 5, just upstream of exon 6. We concluded that the human $GABA_B$ receptor may be regulated by two independent promoters, and not by one single promoter as expected. The first putative promoter, denoted P1a (SEQ ID NO:1) and described in detail below, may regulate transcription of $GABA_BR1a$-type splice variants and the second putative promoter, denoted P1b (SEQ ID NO: 2) and described in detail below, may regulate transcription of $GABA_BR1b$-type splice variants.

As indicated in the schematic representation of P1a and P1b (Figure 2), both putative promoters lacks a TATA box. However P1b has an initiator (Inr) element in position 4375-4381 which is located 24-30 bp upstream of the position corresponding to the 5´ end of the longest known cDNA isolated with "rapid amplification of cDNA ends" (RACE) PCR amplification. The Inr element may therefore direct the start of transcription from P1b. P1a contains neither a TATA or an Inr element and the transcription from R1a may therefore initiate from different start sites which is often the case in promoters lacking TATA boxes or Inr elements. Both P1a and P1b contain multiple GC rich regions at pos. 3009-3016, 3037-3044 and 3116-3123 in R1a and pos. 4080-4087, 4196-4204, 4241-4249 and 4272-4279 in R1b, which are potential binding site for the SP1 family of transcription factors. SP1 binding sites are often found in TATA lacking promoters where they often substantially contribute to transcription. In addition, to the indicated GC sequences in Figure 2 there are also other GC motifs that may function as SP1 binding sites. P1a further

contains an activator protein-1 (AP-1) site at position 1497-1503. AP-1 sites are recognized by AP-1 transcription factors which consists of homodimers of members of the Jun family or Fos/Jun heterodimeric complexes. AP-1 complexes also interact, by protein-protein interactions, with members of the steroid receptor family and are therefore also targets for steroid receptor signaling. In addition to the GC motifs P1b also contain an activator protein-2 (AP-2) site at position 3844-3851 and a cAMP responsive element (CRE) at position 4308-4315. Especially the finding of a consensus CRE (TGACGTCA) is interesting as this promoter element is found in many genes regulated by cAMP which are bound and regulated by members of the ATF/CREB gene family. This sequence may therefore be an important target for cAMP mediated signaling via G-protein coupled receptors, including $GABA_B$ receptors.

We conclude that transcription of the human $GABA_B$ receptor gene may be regulated by two putative promoters, P1a and P1b, that may independently regulate expression of human $GABA_B$ receptor 1a and 1b splice isoforms, respectively.

Example 2. Determination of $GABA_B$ R1 promoter P1a and P1b activity

To experimentally determine if P1a and P1b indeed act as promoters, we fused these regions to a cDNA encoding firefly luciferase to be used as a reporter of promoter activity in transfected cells.

Reporter constructs containing R1a and R1b promoter fragments were generated by PCR using plasmid pAM 364, containing genomic sequence covering the promoter regions, as template. PCR reaction was performed by standard procedure (Perkin Elmer). Briefly, an initial denaturation at 95°C for 4 min was followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, elongation at 72°C for 1 min and finally a 7 min elongation at 72°C. In the sequence for primers used to generate promoter fragments (for details see Table 1. below), a Nhe I and Hind III endonuclease restriction enzyme site was introduced, in 5´and 3´ primers respectively, to enable sub-cloning into Nhe I/Hind III

digested pGL3-Basic luciferase reporter vector (Promega). Hence, complete promoter-reporter constructs (pAM440, pAM438 and pAM436) contain R1a and R1b promoter fragments (indicated size see table) fused to the luciferase reporter gene. Plasmid DNA were purified using Qiagen tip-100 columns according to suppliers instruction. Correct fragment insertion was verified by DNA sequencing.

Table 1. Nucleotide sequence of primers used to generate promoter fragments

Primer No.	Restriction site	Promoter sequence	Position	Sequence 5´-3´
1582	HindIII (AAG CTT)	SEQ ID NO: 1	3440-3424	AAG CTT CTC GGC GCG CGG GCC CG
1583	NheI (GCT AGC)	SEQ ID NO: 1	2341-2362	GCT AGC CAA GAG CTT CTG GAG CCG
1584	NheI (GCT AGC)	SEQ ID NO: 1	720-741	GCT AGC TGT TAC ATG CAG AGC AAT C
1585	HindIII (AAG CTT)	SEQ ID NO: 2	4439-4421	AAG CTT CCT ACG GCC CCC GCG
1586	NheI (GCT AGC)	SEQ ID NO: 2	3321-3340	GCT AGC GCG CAC TGC AAT GCC CTC

To determine putative promoter activity, the reporter gene constructs were introduced into mammalian cells by transfection. In this study the cell line ND7/23 (ECACC Ref No: 92090903) was used. ND7/23 is a hybrid cell line originating from a mouse neuroblastoma (N18tg2) fused by PEG to a rat dorsal root ganglion neuron cell line. This cell line was chosen since it express functional $GABA_B$ receptors as evidenced by radioligand binding studies. Cells were cultured in a 1:1 mixture of Dulbecco´s modified medium (DMEM) and Ham´s F12 medium supplemented with 10% (v/v) fetal bovine serum (FBS). Cells were grown at 37°C in an atmosphere of 5% CO_2. ND7/23 cells ($4x10^5$) were transfected using the DMRIE-C reagent according to manufacturer's protocol (Gibco). Briefly, cells were seeded in 6 well tissue culture plates (Nunc) the day before transfection. Next day, 2μg of promoter-reporter construct and 1μg of transfection control construct (pSV-β-Galactosidase, Promega) was mixed with 0.5 ml Optimem media (Gibco). DNA containing media was then mixed with an equal volume (0.5ml) of Optimem containing 4μl of

DMRIE-C reagent and the combined mixture was then incubated for 45 min. After incubation, the transfection mixture was added to the cells, which were washed with Optimem media just prior to addition of transfection mixture. Following 5h incubation at 37°C, an equal volume of 1:1 mixture of Dulbecco´s modified medium (DMEM) and Ham´s F12 medium supplemented with 20% (v/v) fetal bovine serum (FBS) was added to the cells. Cells were incubated for 24h at 37°C with or without cAMP enhancing supplement as indicated in Figure 3. Before cell harvest, cells were washed in PBS (7.6 mM Na_2HPO_4/NaH_2PO_4 pH 7.4 and 120 mM NaCl), then cell extracts were prepared by addition of 250μl reporter lysis buffer (Promega) to cells, followed by transfer of cell suspension to 1.5 ml tubes. Cells were further lysed by one round of freeze-thawing and 15s of vortex. Cell debris were removed by 2 minutes of centrifugation at 12 000 rpm. Luciferase activity in cell extracts was measured in a Luciferase Assay System (Promega), where 40μl of cell extracts was added to a 96-well plate (Maxisorp, Nunc) and mixed with 50μl of luciferase substrate. Luciferase activity was then measured in a LUMIstar (BMG Lab technologies). As internal control for transfection efficiency, β-Galactosidase (β-Gal.) activity was measured in 96-well plate (Maxisorp, Nunc) using a β-Galactosidase Enzyme Assay (Promega) according to suppliers protocol. As seen in Figure 3, transient transfection of ND7/23 cells shows that both P1a and P1b has promoter activity as pAM438, pAM436 and pAM442 (Figure 3) result in reporter expression, while the pAM440 (vector control) has very low activity. In addition, this experiment demonstrates that especially reporter expression originating from pAM442 (R1b) can be induced by the cAMP activating agent forskolin. Moreover, forskolin induced expression may also be further enhanced in the presence of the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine (IBMX) although this experiment does not show a significant difference.

In conclusion, this experiment demonstrate that P1a and P1b both have promoter activity and that the degree of activity can be modulated using the cAMP activating agent forskoline.

Example 3. Screening for substances modulating P1a and P1b activity

Modulating $GABA_BR1$ expression in a controlled way is a means to regulate signaling through $GABA_B$ receptors which could be of significant therapeutic value for a variety of conditions. Particularly, the ability to specifically regulate expression of either 1a- or 1b-type $GABA_B$ receptor splice isoforms could be of medical value if these isoforms could be attributed to specific conditions.

The experiment presented in Example 2 demonstrates that the use of P1a and P1b promoter/reporter constructions can be used to monitor $GABA_B$ R1 expression in screens for therapeutic agents that can modify the expression of $GABA_B$ receptor 1 isoforms. A screen for P1a and P1b modulating substances could be performed in ND7/23 cells as described in Example 2. A screen for P1a and P1b modulating substances could in addition be performed in any cell type with endogenous expression of $GABA_B$ receptor 1 and 2 isoforms, in cells expressing recombinant $GABA_B$ receptor 1 and 2 isoforms and in intact cells and in extract or fractions of cells expressing endogenous and recombinant proteins modulating $GABA_B$ receptor function. Such screens could furthermore be done in tissues and in living organisms.

Example 4. Functional analysis of $GABA_B$-R1 promoter fragments and modified forms

In order to identify functionally active promoter fragments of P1a and P1b, a deletion analysis of DNA fragments containing promoter fragments can be performed in two steps.

It is anticipated that P1a and P1b comprise active fragments that can mediate increased expression by binding of transcription factors that are activators as well as active fragments which can mediate decreased expression by binding of transcription factors that are repressors.

In the first step, promoter fragments mediating expression of a reporter gene when used in reporter constructs can be stepwise deleted or truncated to identify important regions. Briefly, truncated or deleted promoter fragments are created by PCR using specific primers and the already identified promoter sequences as template. Reporter constructs (as exemplified in this application) comprising the deleted or truncated promoter fragments are then created. These reporter constructs can then be used in transfection experiments, as described above, to identify important regions of the promoters manifested in altered expression from constructs lacking active fragments compared to none-deleted constructs.

In the second step, the exact location and sequence of transcription factor binding sites within active fragments can be determined by PCR technique using specific primers harbouring desired mutations. Such promoter fragments, with specifically mutated promoter regions, can be used in transfection experiments, similar to those described above, to determine the exact sequence of functionally important nuclear factor binding sites within active promoter fragments, manifested in altered expression from constructs with mutated DNA sequence compared to none-mutated control constructs with equal size.

The above mentioned strategy can also be used to identify the specific active promoter fragments which are important for the effect on promoter activity of active substances identified when screening for therapeutic agents regulating $GABA_B$-R1 expression.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1. The human $GABA_B$ receptor gene

The figure shows the organization of the human $GABA_B$ receptor gene. Exons, represented by vertical squares/bars, are numbered (1-23). Translational start and stop sites are

indicated by arrows. Location of human $GABA_B$ receptor 1 promoters P1a (SEQ ID NO: 1) and P1b (SEQ ID NO: 2) are indicated. The extent of human $GABA_B$ receptor genomic sequence cloned in plasmid pAM 364 is indicated by a horizontal bar.

Figure 2. Schematic representation of the P1a and P1b promoters

DNA fragments used to generate reporter constructs, corresponding to the positions in the P1a and P1b promoter sequence, are shown below each promoter. Putative promoter elements in each promoter are indicated. Arrows indicate putative positions for start of transcription.

Figure 3. Determination of $GABA_B$ receptor 1 promoter P1a and P1b activity

ND7/23 cells ($4x10^5$) were transfected with promoter-luciferase constructs as described above. After transfection, cells were cultured in media without supplement (basal) or in the presence of Forskolin (10μM) or Forskolin (10μM) + 1-methyl-3-isobutylxanthine (0.125mM) for 24h. After incubation, cells were harvested and luciferase activity measured. Luciferase activity, minus background, is shown as arbitrary light units measured in 40 μl of cell extracts. Relative values represents the mean±SEM of two individual experiments.

CLAIMS

1. A nucleic acid molecule constituting a human $GABA_B$ receptor 1 promoter P1a, or a functionally equivalent modified form thereof, or an active fragment thereof.

2. A nucleic acid molecule according to claim 1 selected from:
(a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 1;
(b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the polypeptide coding region of a DNA molecule as defined in (a).

3. A nucleic acid molecule constituting a human $GABA_B$ receptor 1 promoter P1b, or a functionally equivalent modified form thereof, or an active fragment thereof.

4. A nucleic acid molecule according to claim 1 selected from:
(a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 2;
(b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the polypeptide coding region of a DNA molecule as defined in (a).

5. A nucleic acid molecule comprising a nucleic acid molecule according to claim 1, in combination with a nucleic acid molecule according to claim 3.

6. A nucleic acid molecule comprising a nucleic acid molecule according to claim 2, in combination with a nucleic acid molecule according to claim 4.

7. A vector transformed with a nucleic acid molecule according to any one of claims 1 to 6.

8. A cultured cell host harboring a vector according to claim 7.

9. An expression system comprising a nucleic acid molecule constituting a human $GABA_B$ receptor 1 promoter P1a, or a functionally equivalent modified form thereof, or an active fragment thereof.

10. An expression system according to claim 9, comprising a nucleic acid molecule selected from:
(a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 1;
(b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the polypeptide coding region of a DNA molecule as defined in (a).

11. An expression system comprising a nucleic acid molecule constituting a human $GABA_B$ receptor 1 promoter P1b, or a functionally equivalent modified form thereof, or an active fragment thereof.

12. An expression system according to claim 11, comprising a nucleic acid molecule selected from:
(a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 2;
(b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the polypeptide coding region of a DNA molecule as defined in (a).

13. An expression system comprising a nucleic acid molecule according to claim 1, in combination with a nucleic acid molecule according to claim 3.

14. An expression system comprising a nucleic acid molecule according to claim 2, in combination with a nucleic acid molecule according to claim 4.

15. An expression system according to any one of claims 9 to 14, which, in addition, comprises a reporter gene.

16. An expression system according to claim 15, wherein the reporter gene is selected from:
(a) the firefly luciferase gene,
(b) the bacterial amphenicol acetyl transferase (CAT) gene,
(c) the β-galactosidase (β-GAL) gene, and
(d) the green fluorescent (GFP) gene.

17. An expression system according to claim 15 or claim 16, wherein the promoter and the reporter gene are positioned so that the expression system of the reporter gene is regulated by the $GABA_B$ receptor 1 promoter.

18. An expression system according to any one of claims 9 to 17, wherein the said nucleic acid molecule is transformed in a vector.

19. An expression system according to claim 18, wherein said vector comprises an origin of replication and/or a dominant selection marker.

20. A host cell transfected with an expression system according to any one of claims 9 to 19.

21. A method of assay for $GABA_B$ receptor promoter activity, comprising the use of a host cell according to claim 20.

22. A method for screening compounds which are modulators of $GABA_B$ receptor 1 transcription, comprising the steps of:
(a) transfecting a cell host with a suitable expression system comprising a nucleic acid molecule constituting a human $GABA_B$ receptor 1 promoter P1a, and/or a

human $GABA_B$ receptor 1 promoter P1b, or functionally equivalent modified forms, or active fragments thereof coupled to a reporter gene;

(b) contacting a test compound with the cell; and

(c) determining whether the test compound modulates the level of expression of the reporter gene.

23. A method according to claim 22, wherein the said expression system is an expression system according to any one of claims 9 to 19.

24. A method according to claim 22, wherein the said the reporter gene is selected from:

(a) the firefly luciferase gene,

(b) the bacterial amphenicol acetyl transferase (CAT) gene,

(c) the β-galactosidase (β-GAL) gene, and

(d) the green fluorescent (GFP) gene.

25. A transgenic non-human animal whose genome comprises an expression system comprising nucleic acid molecules constituting $GABA_B$ receptor promoters P1a and/or P1b, or functionally equivalent modified forms thereof, or active fragments thereof, coupled to a reporter gene.

26. A transgenic non-human animal whose genome comprises an expression system according to any one of claims 9 to 19.

27. A method for the screening of compounds which are modulators of $GABA_B$ receptor 1 transcription, comprising the use of a transgenic non-human animal according to claim 25 or claim 26.

28. A method for the screening of compounds which are modulators of $GABA_B$ receptor 1 transcription, comprising the use of tissues or cells isolated from a transgenic non-human animal according to claim 25 or claim 26.

ABSTRACT

The present invention relates to nucleic acid molecules constituting $GABA_B$ receptor 1 promoters P1a and/or P1b, and to methods for screening for compounds which are modulators of $GABA_B$ receptor 1 transcription, said methods comprising the use of nucleic acid molecules constituting $GABA_B$ receptor P1a and/or P1b promoters.

SEQUENCE LISTING

<110> Astra AB

<120> New methods

<130> H 2174-1 SE

<140>
<141>

<160> 2

<170> PatentIn Ver. 2.0

<210> 1
<211> 3903
<212> DNA
<213> Homo sapiens

<220>
<221> GC_signal
<222> Complement((3009)..(3016))

<220>
<221> GC_signal
<222> (3037)..(3044)

<220>
<221> GC_signal
<222> Complement((3116)..(3123))

<220>
<221> misc_signal
<222> (1497)..(1503)
<223> Pla

<400> 1
gatcatatta atttgaaggt ggcggggcag gatggttctg tggtgcagtt taagattaag 60
aggcatacac cacttagtaa actaatgaaa gcctattgtg aacgacaggg attgtcaatg 120
aggcagatca gattccgatt cgacgggcaa ccaatgaaac agacacacct gcacagttgg 180
aaatggagga tgaagataca attgatgtgt tccaacagca gacgggaggt gtctactgaa 240
aagggaacct gcttctttac tccagaactc tgttctttaa agaccaagat tacattctca 300
attagaaaac tgcaatttgc ttccaccaca tcctgactac taccgtatag ttttctctat 360
tctttcattt ccccccttccc cattccttta ctgtacataa agtaactggt atatgtgcac 420
aagcatatta cttttttttt ttaaaactaa acagccaatg gtatgttttg attgacatca 480
agtggagacg gggggggaaaa tactgattct gtgaaaatac cccctttctc cattagtggc 540
atgctcattc agctcttatc tttatattcc agtaagttat tttgctctca ctgttttaac 600
aacaacaaca aaaaaacaac aacataaaaa tccttgcata ccttgttcaa ttggagaatt 660
ttaatgtttt tcatttatca ttgtaaaacc aaggacaatt ttataacttt tttgtactta 720
gctgttacat gcagagcaat ctgtctttaa gtagggataa attactctaa aacaaaaaag 780
aatcctagat agttttccct tcaagtcaag cgtcttgttg tttaaataaa cttcttgttt 840
aaaaaaaaaa aaagtaaaaa agaaaagtta tgcaacaatt aatggcccag aggcaatcct 900
tgttaacatt ttgatgcatc ttttagctgt tttttttttt tttttttttt ttgactgagt 960
ttgactcttg tcacccaggc tgaagtgcaa tggcatggca tgatcttggc tcactgcaac 1020
ctccgcctcc cgggttcaag tgattctcct gcctcagcct cctgagtagc taggattacg 1080
ggcatgcacc accatgcctg gctaattttg tatttttagt agagttgggg cttctccaca 1140
ctggtcaggc tggtctcgaa ctcccaacct caggtgataa gggaaggggc actattgaca 1200
tttatggttg gggcagaggt gtaagatatt cttcaaagca ctacctacat gttgaagaat 1260
tgttcctcac ccagattctc aaaagtcccc caggacattc acgtagtgaa aacctgtgtt 1320
taattatctg agcctataac ttaatacagt tttaaaattt ttttttaaat atacagtgaa 1380
ctttctagga atgcaattat agttgtgtgt aaaattaggg aaaattaact ttgctaccaa 1440
gagttgttca acattttgtt aaatcacttc attgatggca acatgctgga ggtagttgag 1500
tcaccaactc agcacctgga tcagcctgtg ttggtagcag tttcatcccc gtggttctgt 1560
gaataggtgg aagcatctgc ttactccatc aggacttcta gggtagtcgg gccttggcac 1620
tcacacatta aaatactgtt tatgttattt tattgcaagt tacttttctt tcatttcccc 1680

```
tttacgttac agaaagggaa gcattttgct ttctgtttaa agttgtgtat gtaggtaggt 1740
tatatcatct awgacttttct ctccctcctt ccctttcttt ttgtttgaga tggagtcttg 1800
ctctgtcacc caggctggag tgcagtggtg cgatcttggc tcactgcaac ctctgcctcc 1860
cgggttcaag cgattctggt gtctcagctg ggattacagg cgcacaccat cacaccacgc 1920
taatttttct atttttagta gagatggggt ttcgccatgc tggccaggcc aggctggtct 1980
caaactcctg agctcaagtg atcagtccgc ctcggcctcc caaagttctg ggatttcagg 2040
cgtgagcctc atctatgaat ctcaatttag gacagtaaaa gtgtcattac aaaaatattt 2100
attgtaaaaa agggttggag gttgagaatc tcaattctag tcagtctctc agtgtttggt 2160
ttcttcctac catttttccc cctaggacca gccagaaagc agcttttttt ttgtcccccc 2220
caacaaggag cccactgttt cctctcccag cccaaactca ggcctacgaa caacaacagc 2280
actacacaca cacacacaca cacacacaca cacacacaca caccccctcca cttcaaggta 2340
tagccaagag cttctggagc cgtcaaaaag gtctgtacct gctgtcttta gagcttccag 2400
tttgcccttg gtcaagaaat actgtttgct aggctctgct ggagtacatc aggtaatact 2460
ggcttctaaa ccaccctgag gttcttttct cttgtccttt tactcccttc gtacttcaat 2520
ttctctcctt gatgtccccc tccctgtttt gtttttttgcc tccaatccgt tctgcgcgtt 2580
ccctgcagag caggcgagta gcaatgctgc tggaccatgg agctgctcta gtctcccaga 2640
aatctcttct acacccaacc cttcttgcgc ttaggtggtc ctcagtcccc ctccccccacc 2700
tccttctgac ccaggcttct ttctcgccct ccggtcgcag ttctcctggg catctgcctc 2760
tgcctctctc ctctcacccg gatctagggc tgccttctct ttgtgcagcc gtctttctcc 2820
accttcatcc cagactccct gtctcagcgc cagctcctct gcctttggct cgggttccct 2880
ctccccccacc ccagcttcca gttgtttggc ccgcaggtcc ctcggcagtg accggcgccc 2940
cccgacgagt gcgtgtgcac cagggcacct ccctctcccc cacctctcag ccccgcgcct 3000
ctccaccgcc cgccccaccg cgctgtgggc ggtccagggc ggggctggga tccggggcgg 3060
ctcccggggc tcgggttgtg ggaggcgccc tctccccggt cttcccctct cttccccccg 3120
ccctgccttc ccttgcaccc tccttcttcc ctccgcccgg gagctctccc tggtccccgg 3180
cgccgcctcc ttccctcccg gctccccgct ccccgctccc gtggctgccg ccgcccccggg 3240
gaagaagaga caggggtggg gtttgggggga agcgagagag gaggggagag accctggcca 3300
ggctggagcc tggattcgag gggaggaggg acgggaggag gagaaaggtg gaggagaagg 3360
gagggggggag cggggaggag cggccgggcc tggggccttg aggcccgggg agagccgggg 3420
agccgggccc gcgcgccgag gtaagagcca gggccccggg ttagcagggc tcggagaggg 3480
ggcgcgcggc gtggtggggg agggggcagt gggcgcaggg cccagctggg ggaagcgggg 3540
ctgggggaga ggaggaaccg cggggatgga atcggggagc gctgaggcgg ccgatgccgg 3600
gagcgtgggt aagccaggct tctgcgagcc gcgggggccg ggggagagga ggtggtgaga 3660
ggtggagtcc gggagggttg ggggccgagg gaggcaggag gagggtgggg acaggctttc 3720
tctcctcctc tccccccacc ccgcgcgggg ctccgccccc gcctcctccg cggggcgctc 3780
tcttggtccc caggctgagc ccggtcggag cctgcgaggc aaccggcaag aggtcgagta 3840
gtctccgggt gcgggccgcg ccggcggggc tcggtccagt cctcatggcc gcctctcact 3900
tag 3903
```

<210> 2
<211> 4594
<212> DNA
<213> Homo sapiens

<220>
<221> GC_signal
<222> Complement((4080)..(4087))

<220>
<221> GC_signal
<222> Complement((4196)..(4205))

<220>
<221> GC_signal
<222> Complement((4241)..(4249))

<220>
<221> GC_signal
<222> Complement((4272)..(4279))

<220>
<221> misc_binding
<222> (3844)..(3851)

<223> AP-2

<220>
<221> misc_binding
<222> (4308)..(4315)
<223> CRE

<220>
<221> misc_binding
<222> (4375)..(4381)
<223> Initiator

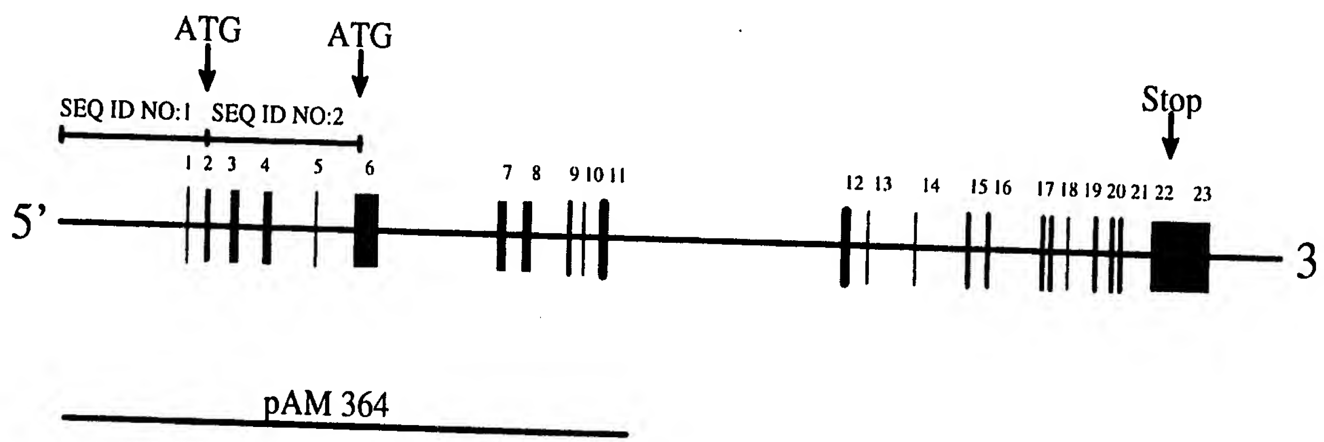
<400> 2

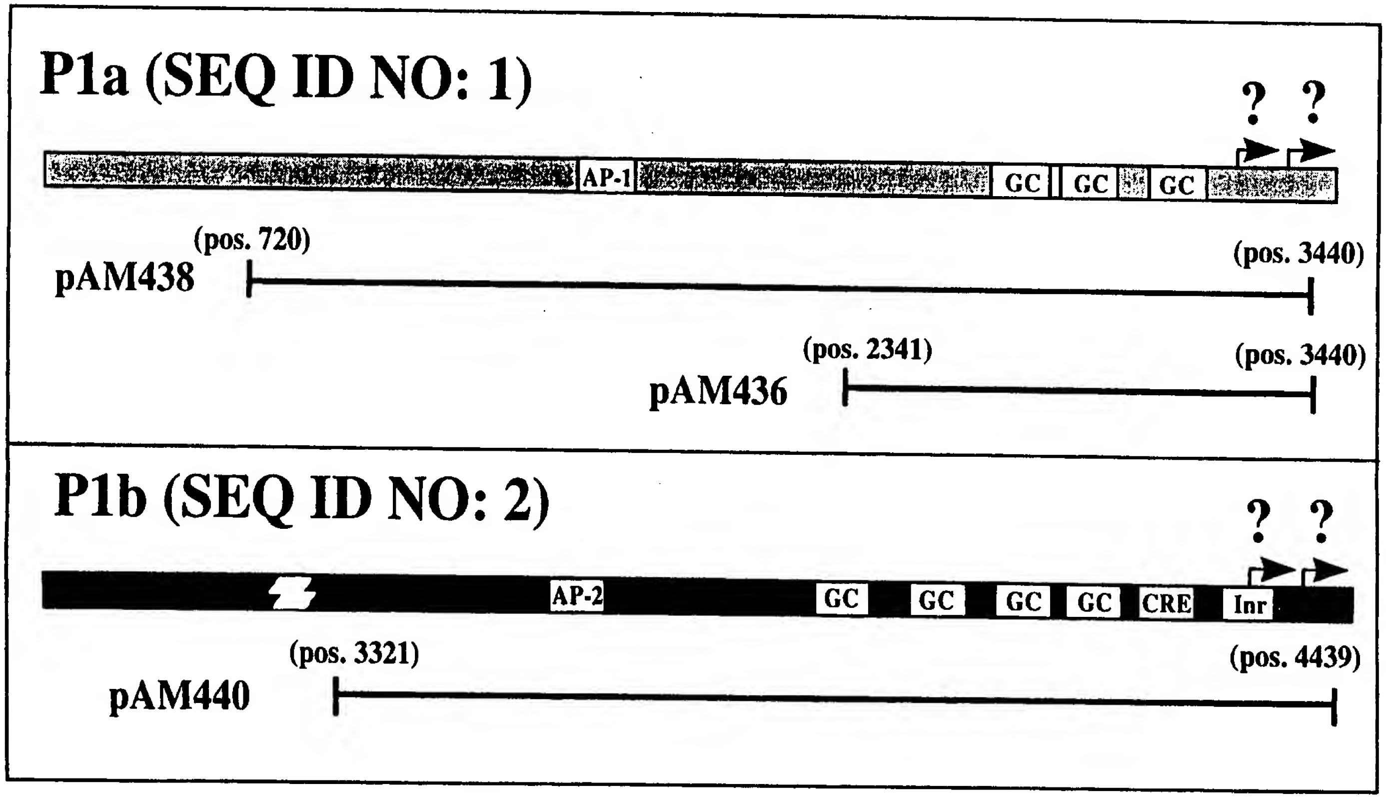
```
atgttgctgc tgctgctact ggcgccactc ttcctccgcc ccccgggcgc gggcggggcg 60
cagaccccca acgccacctc agaaggtgca tccttcttcg acgacctccg gccctccttc 120
gctccacttc cctttccctg catctcctca tttctggtcc tcatcactat cccatcagtc 180
ccacatatca tcccggtctg gcaacccctt ctgctcggcc cgactttact actgctgacc 240
tccttctgtc accccacgtt actatccagc acctcttttc tctgcccaca ttgctacact 300
ataccacctt cctgtgcatt ttctccgcct caatcccctt tcccagcccc acattactac 360
ctcaattact ccctttcttt ggtcccactt tgctgtccag atgatcttat tagcctccct 420
ttatcctcct atcctaattc aactggaata tcctcattta gccttttttt ttaaagaaaa 480
gctccaccca catatcatac ccttcatgat ttcttaatta cttttctttc ttacctccac 540
ccagcaccct tccctcccca cttgtgggtt ctctcatcag ctttaaccct ggccctttac 600
tctctgtcct ttagccaggg gatctgtacc tgtccccact cccaccctct agtgccccat 660
ccctcttcct ctgtccccag cctgcccaca gaccacgccc tactctcccc ttcctcccac 720
tggggagcct gccttttcct ctttcccacc attcctctct gtatgcctcc ccgactcacc 780
ccttaggttg ccagatcata cacccgccct gggaaggggg catcaggtac cggggcctga 840
ctcgggacca ggtgaaggct atcaacttcc tgccagtgga ctatgagatt gagtatgtgt 900
gccgggggga gcgcgaggtg gtggggccca aggtccgcaa gtgcctggcc aacggctcct 960
ggacagatat ggacacaccc agccgctgtg gtgagtagcc tcggaagccc ctcccctctt 1020
caagactatt cctttcctg ccgcaaactt agcattactg cttgcaagtc agcactttaa 1080
atccagtata ccaaaattca caaatacatt tattgaatga ctactacata agagcaattt 1140
tgctctgtgc ggttggaggt agtagagcta gcagcctgca cagttcattt catcctccct 1200
tcattaggcc actgatcatt ggcctataac attgataatt catcttgtca gttattctct 1260
ttgaggatca ttagtggcag atgatgacaa aaaaattcta aaatgatttc atcacatttt 1320
tgaatacctc tgtcaccaac ccagagacca tatgcccaag aaacaaaagc cagtttaata 1380
ttaatagaag ccaactataa taagaaaagc aaatctgatt gtgcatccaa agttatatac 1440
atctacatat ttcaaagcca gagaaccgcc cactgtagct gactttgaag agatcccatt 1500
ttgtgtgctt atagccccat cttgggttcc taaaatggta attttttttt tcttttggga 1560
atgtgtggat gcttgcacag gtaagggagg attggaagat aggtaggcaa atccttttca 1620
catgtgattt tctttagagc aggatgcttg tggacccaaa cctgcacctg agtcccctgc 1680
tctttaaagg gaaagagcct tcttcaactc gcctctcttc ttattttcct atctctccac 1740
agtccgaatc tgctccaagt cttatttgac cctggaaaat gggaaggttt tcctgacggg 1800
tggggacctc ccagctctgg acggagcccg ggtggatttc cggtgtgacc ccgacttcca 1860
tctggtgggc agctcccgga gcatctgtag tcagggccag tggagcaccc ccaagcccca 1920
ctgccagggt gaggggaaca gctgcctgca tgcagctgat gaggacgctt gtgtgaggat 1980
gggagtgggg tgggaatgga taatgggaaa gaatggagag ctataaaaat gtgggggagg 2040
acactggaaa ggggagatga aagtcccttt ttcctccatc acctgcctca aacttcctct 2100
tgcagtcccc ggtatcctct gtaggttggg ggcttccttc ctttaccttt taaaaaaatc 2160
ttcctgctcc cgattcttag acctcacgtt ttctcttttc ctttatgaat ctcacctctc 2220
tcaccttctt caggtttaaa tactccaatt ttccctttct ctaaacttag aaatttccat 2280
gcatcaccct cttctagaat tcatccctca ccattcctta tataattgat ttattgtaaa 2340
gactcagaaa taaatcaaac attctactaa gaaaaattga gaaggggagc tctgggggtg 2400
gaaacatatt agggtaaaag acttaaaatt ggaggcagca ttatcagaag atgaagaaca 2460
actcagggat ggggtgggaa gaagacaggt ccttttctgk acttcctaga caacctccat 2520
tattccctaa gggaatcagt gttgtgtctg tctacytttt tttttttttt tttgccacgt 2580
aattttacaa actctccctt ttctaggcac ccgaactctc tgccatcttc tctcctggga 2640
tgcagtcatc ccatttgtat gcctcatact tcctctaccc tggtagattc tttcaagatc 2700
cttgggcttt actttcctca cataactcag ttattctgct tctagtttac cattttattc 2760
tggaaattga gagtcccatc caggggtgga cttatgacac tactgaaact tagacttcaa 2820
ggttcctcac ctacagggcc ctcttcctgt gctctaataa tatagagggc tcgatggata 2880
tgtgttcata tggtaacagg cttttgtaaa aattgcagaa ataagatttt aacagcaatt 2940
gcttaaagcc aattgtatgt gtaatttttt ttcttaaaga ctcccaattt tgtaatattc 3000
aggcaccaca gaaccaagat ctgccccaaa cttagctatt ggcattcccg tctcaaattc 3060
tgttgtccta tgaaaaatcg aagaagaaaa taagtcctga ccccccttacc cccagaccca 3120
ccttgttctt atccccaggc accctcccct cagaaacgca ggcttctgct ctccccggtc 3180
ttcagcatgg acaggtgtgg gagggggctg gggatcaggc cagggaagct gggcgccagt 3240
ggtaactctt ctctgatccc cgtctttcct gctgccagtg aatcgaacgc cacactcagg 3300
tgagatgaga aacccttacc gcgcgcactg caatgccctc cccttcactc tgcaccctcc 3360
```

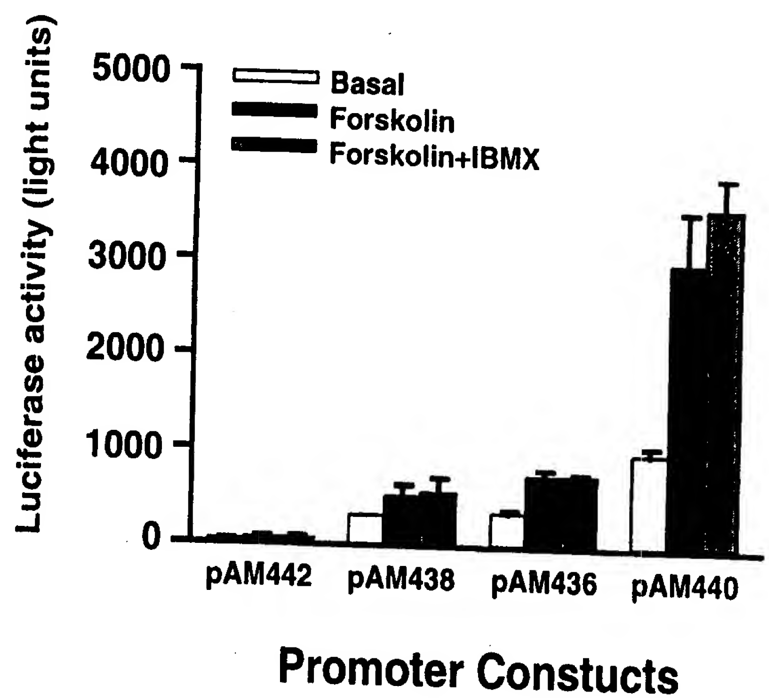
acccccctga aattctgccc ttaggctacg gggcgtcgtc ctttcgcacc ttccccaacc 3420
caccccagtt tgcggccacc cccttccctc cctacctgtt tcctgcctcc agtcccggtt 3480
ttccacgagg ctgcggtctc tccttgtccc tgcttggcta cacttccctg ggctccacct 3540
cctcccagac tgagcctcgc cggtgtcagg cagagcccag cagarggcgg cagggtgctg 3600
ggagaccctg agctcccacc acgttttccc ctgtggggtt ccttgcgacc ttcgctggaa 3660
ccttttccag cctgctgcct cctaggattt cacctaatgg actttctcag cctgtcccac 3720
ccatcccaac cctggccagg cctctcgcgc tcttccccac atcttttcct tccgtgtacc 3780
ccttccctcg tcttttctca attccatgtc ctgtctccct ttcttaggct tctgtctacc 3840
cagccccagg ctcccttcca cgaccccacc actccctcaa accagcctcc cttccgtacc 3900
caactcgttc cctccaaaac cgtttcctct cccccacatc ctcagtgctt cactgtatcg 3960
actcatactc ccacttcaga cctcaggcgc cagccccgtt tctctcccgt cccactcgca 4020
tccttccctt cctaccctgg ttcctccgtg cttcagcctc ccgcggctcc ctccgcccac 4080
cccgccctcc tggcacgccc cgtccccatt tctcctcccc tcgggtcccc ttaagtgaga 4140
tccctccctt cctctttcgt tcctttcctc ctcgaggttg catccccccct cccctccccg 4200
cccctccgac tgtcgctccc acctcggcgc tcgcttccct ccccgccccc ttcctgcctc 4260
cccagctccc gcccgccccc ccaccccccg ctgccgcgcg ccgcccgtga cgtcagagcc 4320
ccctcccagc cccacatctc cctcctgctc ctcctcctcc cctccgtcgg tcagtcagtc 4380
cgcgaggaga gtccgcggtg gcggcgacgg tggcgagagc cgcgggggcc gtaggaagcc 4440
aaccttccct gcttctccgg ggccctcgcc ccctcctccc cacaaaatca gggatggagg 4500
cgcctccccg gcaccctctt agcagccctc cccgggaaaa gtgtcccccc tgagctccta 4560
acgctcccca acagctaccc ctgcccccca cgcc 4594

FIGURES

1/3


ATG
ATG
SEQ ID NO:1
SEQ ID NO:2
Stop
1 2 3 4 5 6
7 8 9 10 11
12 13 14 15 16 17 18 19 20 21 22 23
5'
3'
pAM 364


P1a (SEQ ID NO: 1)
? ?
AP-1
GC
GC
GC
(pos. 720)
pAM438
(pos. 3440)
(pos. 2341)
pAM436
(pos. 3440)
P1b (SEQ ID NO: 2)
? ?
AP-2
GC
GC
GC
GC
CRE
Inr
(pos. 3321)
pAM440
(pos. 4439)


Luciferase activity (light units)
5000
4000
3000
2000
1000
0
Basal
Forskolin
Forskolin+IBMX
pAM442
pAM438
pAM436
pAM440
Promoter Constucts